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Structural analysis of the α-D-glucan (EPS35-5) produced by the *Lactobacillus reuteri* strain 35-5 glucansucrase GTFA enzyme

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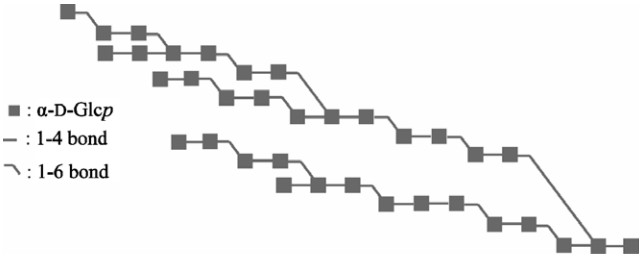
Abstract—The neutral exopolysaccharide **EPS35-5** (reuteran) produced from sucrose by the glucansucrase GTFA enzyme from *Lactobacillus reuteri* 35-5 was found to be a (1→4,1→6)-α-D-glucan, with no repeating units present. Based on linkage analysis and 1D/2D ^{1}H and ^{13}C NMR spectroscopy of intact **EPS35-5**, as well as MS and NMR analysis of oligosaccharides obtained by partial acid hydrolysis and enzymatic hydrolysis, using pullulanase M1 (*Klebsiella planticola*), of **EPS35-5**, a composite model, that includes all identified structural elements, was formulated as follows:



Keywords: α-D-Glucan; Polysaccharide structural analysis; GTFA Glucansucrase; *Lactobacillus reuteri* 35-5; Reuteran; ^{1}H NMR spectroscopy

1. Introduction

The use of lactic acid bacteria (LAB) in food production is commonplace, due to their status as generally recognised as safe (GRAS). One of the reasons for the use of LAB in food (especially dairy) is the generation of large exopolysaccharides (EPSs) in the food medium. The physico-chemical properties of the EPSs lie at the basis of their clearly defined functions in food as stabilisers, gelling agents and thickeners.[1] Whereas in vivo the function of these EPSs is not completely clear, they seem to be involved in adhesion,[2] certain cellular processes[3] and protection against dehydration, phagocytosis or toxins.[3]

Until recently, EPSs from LAB that were analysed were generally heteropolysaccharides with repeating-unit structures.[4] Structures of LAB homopolysaccharides produced by specific glucansucrase enzymes have not been extensively analysed. Only initial structural

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studies have been performed on dextrans (polysaccharides containing mostly (α1→6) linkages) with (α1→2), (α1→3) and (α1→4) branches,[5,6] or linear homopolysaccharides.[7] Recently, a structural-reporter-group concept for α-glucans was established,[8] and applied to the analysis of an α-glucan produced by the glucansucrase GTF180 enzyme, resulting in a detailed composite model.[9]

Previous studies of GTFA, a glucansucrase enzyme with large similarity to GTF180, revealed that it produces **EPS35-5** (reuteran), an α-glucan with (α1→4) and (α1→6) glycosidic linkages.[10] Here, we describe the structural analysis of this homopolysaccharide, using the structural-reporter groups established previously,[8] and enhanced by application on **EPS180**.[9] The various data were combined to formulate a composite model, that includes all structural features identified in **EPS35-5**.

2. Results

2.1. Composition of EPS35-5

Monosaccharide analysis of **EPS35-5** revealed the presence of glucose only and a carbohydrate content of 100% (w/w). Methylation analysis of **EPS35-5** showed the presence of terminal, 4-substituted, 6-substituted and 4,6-disubstituted glucopyranose in a molar percentage of 14%, 46%, 26% and 14%. 1D ^{1}H NMR spectroscopy (Fig. 1) showed an α-anomeric configuration for all glucose residues. Of these residues, 58% is involved in (α1→4) linkages ($\delta_{\text{H-1}}$ ~5.36 and ~5.33) and 42% in (α1→6) linkages ($\delta_{\text{H-1}}$ ~4.96), which is in agreement with the linkage ratios found by methylation analysis (see also Ref. 10). The (α1→4) anomeric ^{1}H signal is split into two overlapping broad peaks, indicating that (-)α-D-Glc*p*-(1→4)-[‡] residues in **EPS35-5** exist in at least two significantly different structural elements. The (α1→6) anomeric ^{1}H signal is represented by a single peak, which may reflect a more uniform structural element for the (-)α-D-Glc*p*-(1→6)- residues.

2.2. Partial acid hydrolysis

After the evaluation of various partial acid hydrolysis conditions, making use of MALDI-TOF-MS and 1D ^{1}H NMR spectroscopy, a large batch of **EPS35-5** (500 mg) was subjected to partial acid hydrolysis with 0.5 M TFA (2 h, 90 °C). The latter conditions rendered a pool of oligosaccharides in a broad distribution of degrees of polymerisation, as indicated by MALDI-TOF-MS (data not shown), 1D ^{1}H NMR spectroscopy (data not shown) and HPAEC analysis on CarboPac PA-100 (Fig. 2A). The glycan pool was pre-fractionated on Bio-Gel P-4, yielding four subpools, denoted by **I**–**IV**. The fragment size distribution in each subpool was determined by MALDI-TOF-MS (data not shown). Subpool **I** consisted of fragments smaller than three Glc units. Subpool **II** contained fragments of three up to five Glc residues. Subpool **III** consisted of fragments of five up to seven Glc units. Finally, subpool **IV** consisted of fragments of seven residues and larger. The fragments in subpool **IV** were mainly too large for full structural analysis, and were not studied further. Subpools **I**–**III** were subfractionated by HPAEC on CarboPac PA-1, using a 0–300 mM NaOAc gradient in 100 mM NaOH (Fig. 2B), and fractions **1** and **2** were isolated from subpool **I**, fractions **3**–**5** from subpool **II** and fractions **6** and **7** from subpool **III**.

2.2.1. Fraction 1. Profiling of fraction **1** on CarboPac PA-100 showed one peak. The 1D ^{1}H NMR spectrum of fraction **1** corresponded with that of isomaltose, α-D-Glc*p*-(1→6)-D-Glc*p* (Scheme 1).[8]

2.2.2. Fraction 2. Analysis on CarboPac PA-100 revealed that fraction **2** consisted of one compound. The 1D ^{1}H NMR spectrum corresponded with that of maltose, α-D-Glc*p*-(1→4)-D-Glc*p* (Scheme 1).[8]

2.2.3. Fraction 3. Analysis of fraction **3** on CarboPac PA-100 showed two component peaks. Fraction **3** was further separated on CarboPac PA-1, isocratically eluted with 100 mM NaOAc in 100 mM NaOH (Fig. 2C), yielding one major fraction **3a**. The MALDI-TOF mass spectrum of fraction **3a** revealed an $[\text{M+Na}]^+$ pseudomolecular ion at *m*/*z* 527, corresponding with Hex$_3$. The 1D ^{1}H NMR spectrum of **3a** (Fig. 3A) showed five anomeric signals at δ 5.412 (**D** H-1, $^3J_{1,2}$ 3.7 Hz), 5.240 (**Rα** H-1, $^3J_{1,2}$ 3.8 Hz), 4.958 (**B**$_\beta$ H-1, $^3J_{1,2}$ 3.8 Hz), 4.950 (**B**$_\alpha$ H-1, $^3J_{1,2}$ 3.8 Hz) and 4.669 (**Rβ** H-1, $^3J_{1,2}$ 8.1 Hz). Starting from the anomeric signals in the 2D ^{1}H–^{1}H TOCSY spectrum (not shown), all non-anomeric signals could be assigned (Table 1). The **Rα** and **Rβ** H-1 values correspond with those found for the -(1→6)-D-Glc*p* unit of isomaltose, which was established as a structural-reporter-group signal for this type of unit.[8] This was further confirmed by the **R** H-5 (δ 4.00 for **Rα** and δ 3.64 for **Rβ**) and H-6b (δ 4.01 for **Rα** and δ 3.93 for **Rβ**) resonances.[8] Taking into account the ROESY inter-residual cross-peak between **D** H-1 and **B** H-4 (not shown), the set of H-3 and H-4 signals of residue **B** at δ 4.01 and 3.66, respectively, indicated a -(1→4)-α-D-Glc*p*-(1→6)- unit for residue **B**.[8] The

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[‡] For Glc residues at semi-defined places in the structure (-)α-D-Glc*p*-(1→*x*)- or -(1→*x*)-α-D-Glc*p*(-) is used. When the structural context of the residue is precisely known, this is indicated as follows: -(1→*x*)-α-D-Glc*p*-(1→*y*)- describing an *x*-substituted residue with an (1→*y*) linkage. In the case of a non-reducing terminal residue α-D-Glc*p*-(1→*x*)- is used, a reducing terminal residue is indicated with -(1→*x*)-D-Glc*p*.

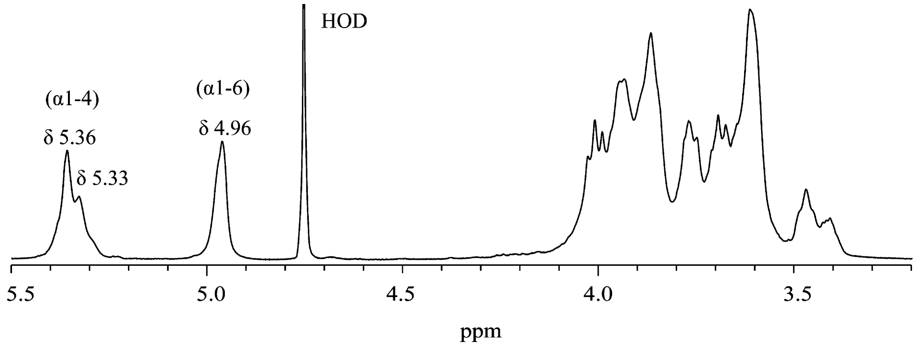


Figure 1. 500-MHz 1D ^{1}H NMR spectrum of intact **EPS35-5** recorded at 300 K in D_2O.

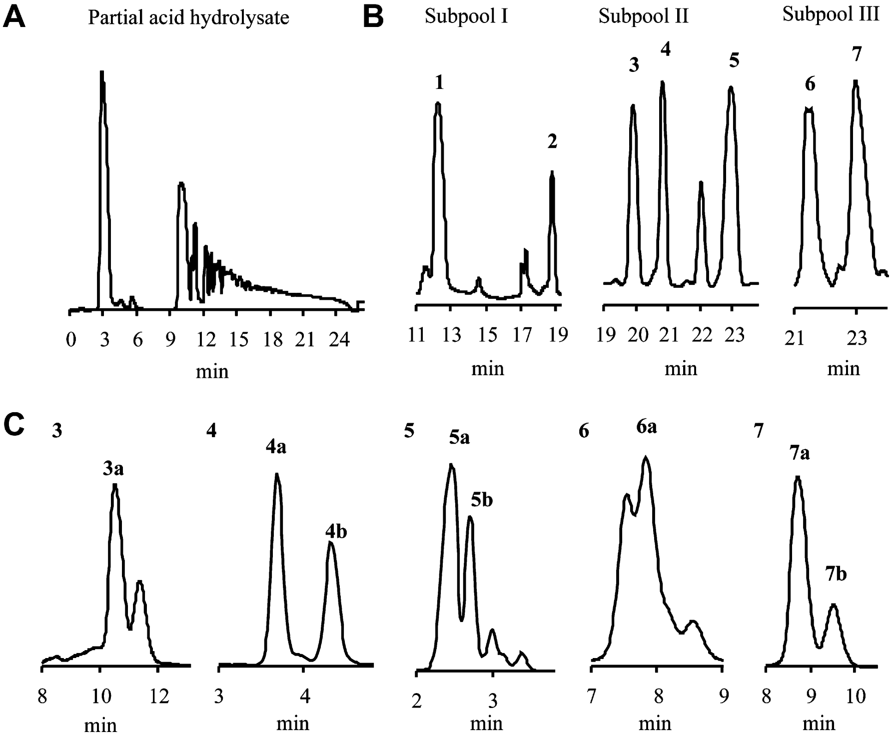
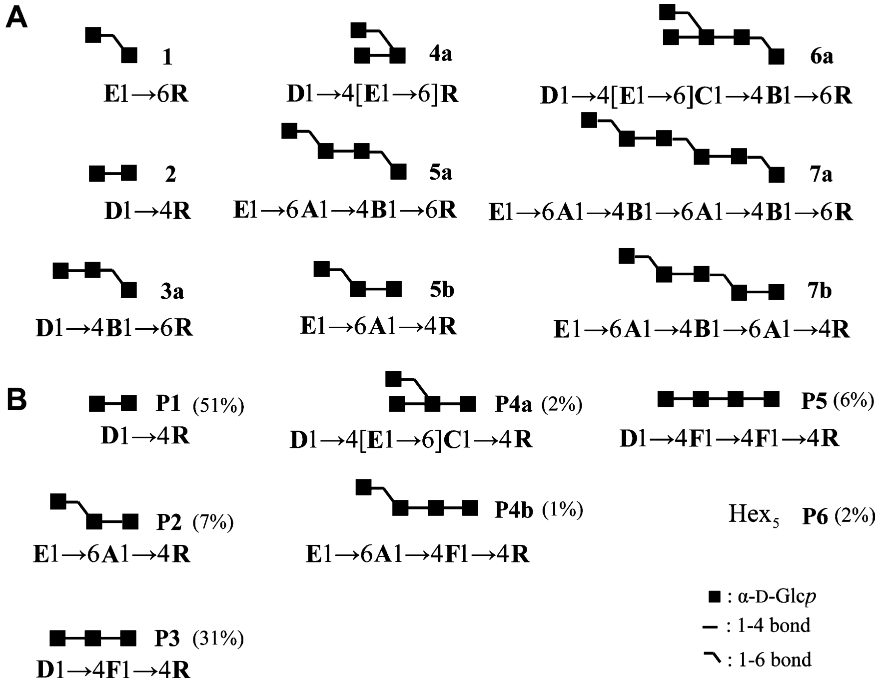


Figure 2. (A) HPAEC-PAD profile of **EPS35-5** partial acid hydrolysate on CarboPac PA-100; (B) HPAEC-PAD profiles of Bio-Gel P-4 subpools **I**, **II** and **III** on CarboPac PA-1, using a linear gradient; and (C) HPAEC-PAD profiles of HPAEC fractions **3**–**7** on CarboPac PA-1, using isocratic conditions. For experimental details, see Section 4.

most notable signal on the **D** H-1 track of the TOCSY spectrum is the H-4 signal at δ 3.417, which was identified as a marker for a terminal α-D-Glc*p*-(1→*x*)- unit;[8] the H-1 value of **D** indicates the occurrence of an α-D-Glc*p*-(1→4)- unit. These data lead to an isopanose structure **D**1→4**B**1→6**R**, that is, α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→6)-D-Glc*p* (Scheme 1).

2.2.4. Fraction 4. The MALDI-TOF mass spectrum of fraction **4** showed an $[M+Na]^+$ pseudomolecular ion at *m*/*z* 527, corresponding with Hex_3. Fraction **4** was further separated on CarboPac PA-1 (eluent: 100 mM NaOAc in 100 mM NaOH) (Fig. 2C), yielding major fraction **4a** and minor fraction **4b** (non-carbohydrate contaminant).

The 1D ^{1}H NMR spectrum of **4a** (Fig. 4) showed four anomeric ^{1}H signals at δ 5.354/5.344 (**D** H-1, $^3J_{1,2}$ 3.9 Hz), 5.222 (**Rα** H-1, $^3J_{1,2}$ 3.6 Hz), 4.985/4.976 (**E** H-1, $^3J_{1,2}$ 3.8 Hz) and 4.660 (**Rβ** H-1, $^3J_{1,2}$ 8.1 Hz). Analysis of the 2D ^{1}H–^{1}H TOCSY spectrum (Fig. 4/180 ms) yielded all chemical shifts of the non-anomeric protons for residues **D**, **E** and **R** (Table 1). In the absence of (α1→2) linkages, both the **D** H-4 (δ 3.41) and **E** H-4 (δ 3.43) values are indicative of a terminal α-D-Glc*p*-(1→*x*)- residue. Taking into account the methylation analysis data of **EPS35-5** (Section 2.1), **D** H-4 fits best



Scheme 1. Structures of oligosaccharide fragments, obtained by (A) partial acid hydrolysis and (B) pullulanase M1 hydrolysis of **EPS35-5**. Quantities determined for the oligosaccharides obtained by pullulanase M1 hydrolysis are included between brackets.

with the occurrence of an α-D-Glc*p*-(1→4)- unit and **E** H-4 with that of an α-D-Glc*p*-(1→6)- unit.[8] The chemical shifts of residue **R** H-3 (**Rα** δ 3.99; **Rβ** 3.78) are indicative of a 4-substitution, confirming the occurrence of a **D**1→4**R** structural element,[8] which is further supported by the **D** H-1,**R** H-4 cross-peak in the 2D ROESY spectrum (Fig. 4). In the case of a 4-substitution, the H-5 signal is to be expected at $\delta \sim 3.94$ for **Rα** and $\delta \sim 3.63$ for **Rβ**, however, in **4a** the H-5 signal is found at δ 4.11 for **Rα** and δ 3.89 for **Rβ**, indicating the additional effect of a 6-substitution.[8] The occurrence of the **E**1→6**R** structural element is further supported by the H-6b chemical shifts of **R**. These data lead to a **D**1→4[**E**1→6]**R** structure for compound **4a**, that is, α-D-Glc*p*-(1→4)-[α-D-Glc*p*-(1→6)-]D-Glc*p* (Scheme 1). The splitting of the anomeric signal of the α-D-Glc*p*-(1→6)- unit is also observed in isomaltose.[8] The anomeric splitting observed across the (α1→4) bond in residue **D** had not been observed previously.

It should be noted that the 4,6-disubstitution of **R** yields a different set of **Rα** H-1 and **Rβ** H-1 chemical shift values. Also the **Rα** H-5 resonance at δ 4.11 can be considered as a new structural-reporter-group signal. These findings lead to a further fine-tuning of the structural-reporter-group concept, similar to the new structural reporters established for **EPS180**.[9]

2.2.5. Fraction 5. MALDI-TOF-MS analysis of fraction 5 revealed [M+Na]$^+$ pseudomolecular ions at m/z 527 and 689, corresponding with Hex$_3$ and Hex$_4$, respectively. Fraction 5 was further separated on CarboPac PA-1 (eluent: 100 mM NaOAc in 100 mM NaOH) (Fig. 2C), rendering a major fraction **5a** and a minor fraction **5b**.

MALDI-TOF-MS analysis of **5a** revealed an [M+Na]$^+$ pseudomolecular ion at m/z 689, corresponding with Hex$_4$. The 1D ^{1}H NMR spectrum of **5a** (Fig. 3B) showed anomeric signals at δ 5.404 (**A** H-1, $^3J_{1,2}$ 3.8 Hz), 5.241 (**Rα** H-1, $^3J_{1,2}$ 3.6 Hz), 4.957 (**B** H-1 and **E** H-1, $^3J_{1,2}$ 3.4 Hz) and 4.669 (**Rβ** H-1, $^3J_{1,2}$ 8.1 Hz). Starting from the anomeric signals in the 2D ^{1}H–^{1}H TOCSY spectrum (not shown), all chemical shifts of the non-anomeric protons could be determined (Table 1). The chemical shift positions of H-1α and H-1β of the reducing residue **R** at δ 5.241 and 4.669, respectively, are in agreement with the occurrence of a -(1→6)-D-Glc*p* unit.[8,9] This is further reflected by the **Rα** and **Rβ** H-5 resonances at δ 4.00 and 3.65, respectively (library data: δ 3.99-4.01 and 3.63-3.65).[8,9] The **A** H-1 value at 5.404 ppm is indicative of an (-)α-D-Glc*p*-(1→4)- unit.[8] On the **A** H-1 track the set of H-5, H-6a and H-6b is observed at δ 3.90, 3.72 and 3.97, respectively, corresponding with a -(1→6)-α-D-Glc*p*-(1→4)- unit (compare with panose),[8] leading to the occurrence of a **X**1→6**A**1→4**Y** element.

The overlapping anomeric signals of residues **B** and **E**, indicative of (-)α-D-Glc*p*-(1→6)- units, form a single track in the TOCSY spectrum. However, the difference between the two sets of H-2, H-3 and H-4 can clearly be observed in the built-up series of mixing times (data not shown). The chemical shifts of the set of **B** H-2, H-3 and H-4 at δ 3.60, 3.99 and 3.65, respectively,

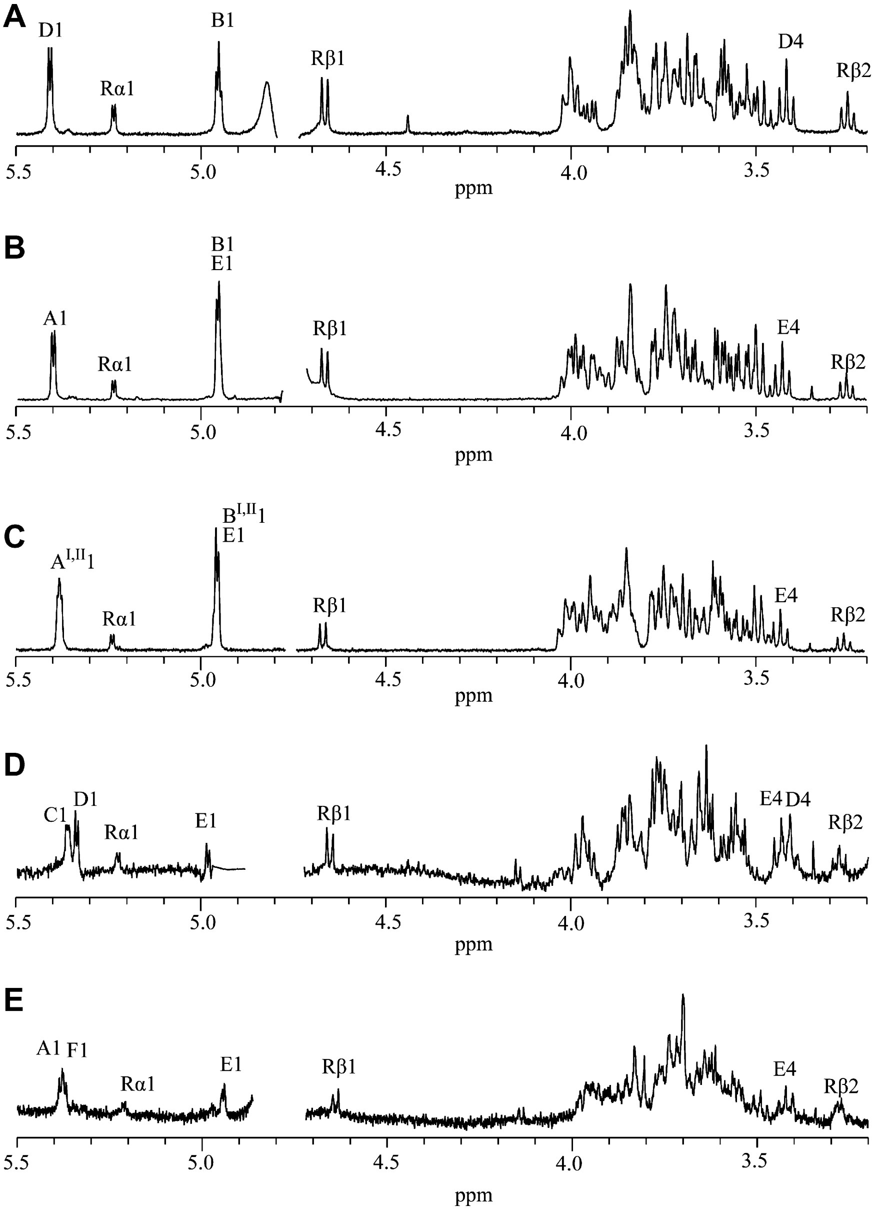


Figure 3. 500-MHz 1D ^1H NMR spectra of (A) fraction **3a**, (B) fraction **5a**, (C) fraction **7a**, (D) compound **P4a** and (E) compound **P4b**, recorded at 300 K in D_2O. Fractions **3a**, **5a** and **7a** were obtained by partial acid hydrolysis of **EPS35-5**, and compounds **P4a** and **P4b** were obtained by pullulanase M1 hydrolysis of **EPS35-5**.

are indicative of the occurrence of a -(1→4)-α-D-Glc*p*-(1→6)- unit (see compound **3a**),[8] leading to an **X**1→6**A**1→4**B**1→6**Z** structure. The presence of **E** H-4 at δ 3.427 (dd, 1H) reflects the occurrence of one terminal α-D-Glc*p*-(1→6)- unit.[8] Considering that **E** is a terminal residue and **R** the reducing residue leads unequivocally to the conclusion that compound **5a** has the structure **E**1→6**A**1→4**B**1→6**R**, that is, α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→6)-D-Glc*p* (Scheme 1).

The structure established for tetrasaccharide **5a** was verified by 2D ^1H–^1H ROESY measurements (not shown), showing inter-residual cross-peaks between **A** H-1 and **B** H-4, **B** H-1 and **R** H-6a, and **E** H-1 and **A** H-6a. It should be noted that the ^1H NMR data of **E**1→6**A**1→4 perfectly fit those observed in the **E**1→6**A**1→4 element of panose.[8]

The MALDI-TOF mass spectrum of **5b** revealed an $[M+Na]^+$ pseudomolecular ion at *m*/*z* 527, corresponding with Hex_3. The 1D ^1H NMR spectrum matches that of panose,[8] that is, α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc*p* (Scheme 1).

2.2.6. Fraction 6.

MALDI-TOF-MS analysis of fraction **6** gave rise to a single $[M+Na]^+$ pseudomolecular ion at *m*/*z* 851, corresponding with Hex_5. Fraction **6**

Table 1. ^{1}H chemical shifts of oligosaccharide fragments of **EPS35-5** obtained by partial acid hydrolysis and of intact **EPS35-5**, measured at 300 K in D_2O

	3a	**4a**	**5a**	**6a**	**7a**	**7b**	**EPS35-5**
Rα-1	5.240	5.222	5.241	5.241	5.239	5.225	—
Rα-2	3.55	3.59	3.54	3.55	3.55	3.58	—
Rα-3	3.70	3.99	3.67	3.71	3.70	4.01	—
Rα-4	3.52	3.67	3.50	3.50	3.50	n.d.	—
Rα-5	4.00	4.113	4.00	4.00	4.00	n.d.	—
Rα-6a	3.73	3.84	3.73	3.72	3.73	n.d.	—
Rα-6b	4.01	4.01	4.03	4.01	4.01	n.d.	—
Rβ-1	4.669	4.660	4.669	4.670	4.669	4.649	—
Rβ-2	3.253	3.285	3.253	3.257	3.255	3.271	—
Rβ-3	3.48	3.78	3.46	3.48	3.49	3.77	—
Rβ-4	3.51	3.67	3.51	3.51	3.53	3.66	—
Rβ-5	3.64	3.89	3.65	3.64	3.63	3.63	—
Rβ-6a	3.75	3.78	3.80	3.76	3.77	n.d.	—
Rβ-6b	3.927	3.95	3.97	3.96	3.96	n.d.	—
A-1	—	—	5.404	—	5.387/80	5.381	5.36
A-2	—	—	3.60	—	3.60	3.60	3.60
A-3	—	—	3.68	—	3.70	3.70	3.70
A-4	—	—	3.496	—	3.492	3.497/92	3.48
A-5	—	—	3.90	—	3.92	3.92	n.d.
A-6a	—	—	3.72	—	3.75	3.74	3.74
A-6b	—	—	3.97	—	3.97	3.96	3.94
B-1	4.958/50	—	4.957	4.960/51	4.955	4.954	4.96
B-2	3.60	—	3.60	3.61	3.60	3.61	3.61
B-3	4.01	—	3.99	4.01	4.01	4.00	4.01
B-4	3.66	—	3.65	3.65	3.66	3.65	3.65
B-5	3.85	—	3.85	3.85	3.85	3.85	3.85
B-6a	3.86	—	3.85	3.86	3.86	3.87	3.89
B-6b	3.80	—	3.82	3.83	3.82	3.84	3.85
C-1	—	—	—	5.361	—	—	5.33
C-2	—	—	—	3.65	—	—	3.65
C-3	—	—	—	3.98	—	—	3.98
C-4	—	—	—	3.67	—	—	3.65
C-5	—	—	—	4.05	—	—	4.05
C-6a	—	—	—	n.d.	—	—	3.91
C-6b	—	—	—	3.97	—	—	3.97
D-1	5.412	5.354/44	—	5.341	—	—	5.36
D-2	3.59	3.58	—	3.59	—	—	3.59
D-3	3.70	3.71	—	3.70	—	—	3.70
D-4	3.417	3.410	—	3.418	—	—	3.42
D-5	3.75	3.76	—	3.75	—	—	3.75
D-6a	3.86	3.86	—	3.86	—	—	n.d.
D-6b	3.78	3.77	—	3.77	—	—	3.77
E-1	—	4.985/76	4.957	4.986	4.955	4.954	4.96
E-2	—	3.54	3.56	3.55	3.56	3.57	3.56
E-3	—	3.75	3.72	3.76	3.75	3.75	3.75
E-4	—	3.431	3.427	3.435	3.432	3.429	3.43
E-5	—	3.74	3.73	3.75	3.75	3.74	3.75
E-6a	—	3.85	3.85	n.d.	3.85	3.85	3.85
E-6b	—	3.76	3.74	3.77	3.77	3.75	3.75
F-1	—	—	—	—	—	—	5.33
F-2	—	—	—	—	—	—	3.60
F-3	—	—	—	—	—	—	3.98
F-4	—	—	—	—	—	—	3.65
F-5	—	—	—	—	—	—	3.97
F-6a	—	—	—	—	—	—	n.d.
F-6b	—	—	—	—	—	—	n.d.

Residue labels correspond to those used in Scheme 1.

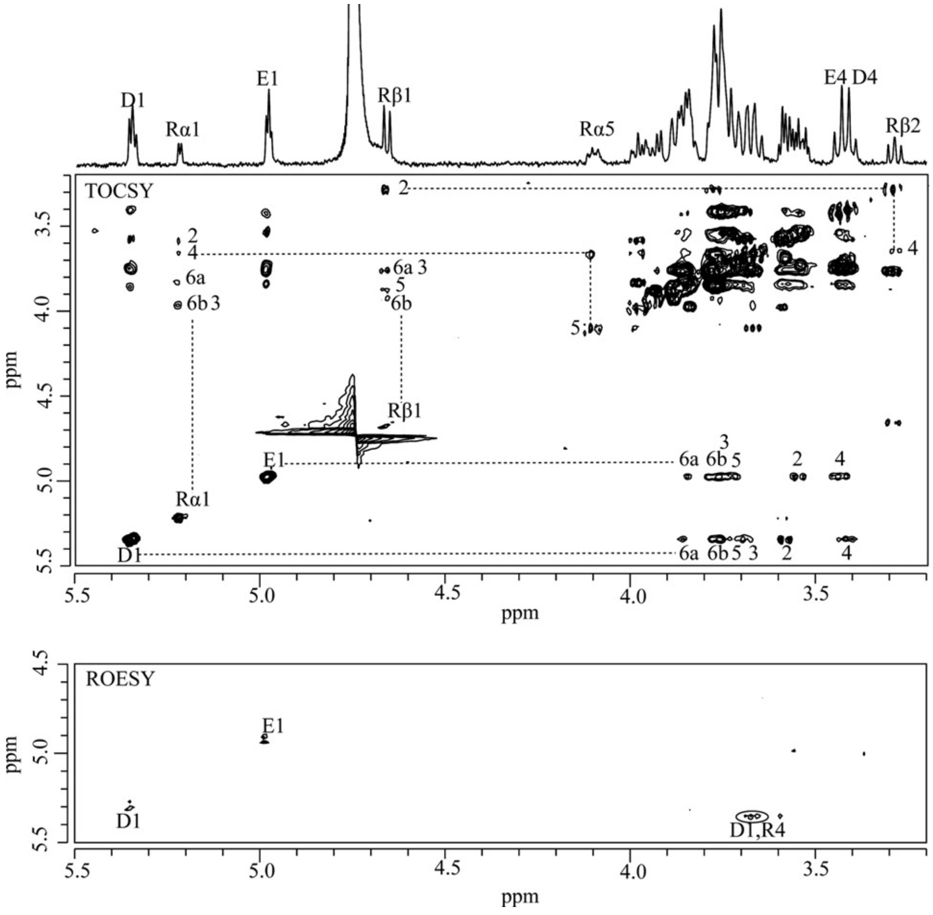


Figure 4. 500-MHz 1D ^{1}H NMR spectrum, 2D ^{1}H–^{1}H TOCSY spectrum (mixing time 180 ms) and 2D ^{1}H–^{1}H ROESY spectrum (mixing time 300 ms) of fraction **4a**, recorded at 300 K in D_2O. Anomeric protons in the TOCSY spectrum (Rα1, etc.) have been indicated on the diagonal; numbers in the horizontal and vertical tracks belong to the cross-peaks of the scalar-coupling network of the residues indicated. In the ROESY spectrum inter-residual couplings (D1,R4 means a cross-peak between **D** H-1 and **R** H-4, etc.) have been indicated with circles.

was further separated on CarboPac PA-1 under isocratic conditions (Fig. 2C), yielding one major fraction **6a** (Hex_5).

The 1D ^{1}H NMR spectrum of **6a** (Fig. 5) revealed six anomeric signals at δ 5.361 (**C** H-1, $^3J_{1,2}$ 3.8 Hz), 5.341 (**D** H-1, $^3J_{1,2}$ 3.6 Hz), 5.241 (**Rα** H-1, $^3J_{1,2}$ 3.8 Hz), 4.986 (**E** H-1, $^3J_{1,2}$ 3.6 Hz), 4.960/4.951 (**B** H-1, $^3J_{1,2}$ 3.6 Hz) and 4.670 (**Rβ** H-1, $^3J_{1,2}$ 7.9 Hz). The chemical shifts of the non-anomeric protons, as included in Table 1, were obtained from 2D TOCSY spectra with increasing mixing times (Fig. 5/180 ms). The chemical shift values of **Rα** and **Rβ** H-1 at δ 5.241 and 4.670, respectively, correspond with the values established for the occurrence of a -(1→6)-D-Glc*p* unit.[8] The δ values of **Rα** H-5 at 4.00 ppm and **Rβ** H-5 at 3.64 ppm confirm the 6-substitution of residue **R**.[8] The H-1 signal of residue **B**, split into $\mathbf{B}_\alpha$ and $\mathbf{B}_\beta$ at δ 4.951 and 4.960, respectively, corresponds with an (-)α-D-Glc*p*-(1→6)- unit, influenced by the reducing residue anomeric configuration, suggesting a **B**1→6**R** element. The set of **B** H-2, H-3 and H-4 at δ 3.61, 4.01 and 3.65, respectively, revealed that **B** is a -(1→4)-α-D-Glc*p*-(1→6)- unit (see also compounds **3a** and **5a**). In a similar way as discussed for compound **4a**, the **D** H-4 (δ 3.418) and **E** H-4 (δ 3.435) chemical shifts indicate the presence of a terminal α-D-Glc*p*-**D**-(1→4)- and a terminal α-D-Glc*p*-**E**-(1→6)- unit, respectively. In fact, the **D** and **E** H-1 signals at δ 5.341 and 4.986, respectively, match those of the **D** and **E** residues in **4a**, occurring in a **D**1→4[**E**1→6]**X** element. Taking into account that **EPS35-5** only contains (α1→4) and (α1→6) linkages, the **C** H-1 value (δ 5.361) has to be correlated with an (α1→4)-linked residue. Moreover, the set of **C** H-2, H-3 and H-4 values indicates a -(1→4)-α-D-Glc*p*-(1→4)- unit. The H-5 signal, however, is found at δ 4.05 (library maltotriose residue **B**: $\delta_{\text{H-5}}$ 3.85),[8] being in favour of an additional 6-substitution (see compound **4a**), and leading to the conclusion that residue **C** is a -(1→4,6)-α-D-Glc*p*-(1→4)- unit. Taken together, these data lead to a **D**1→4[**E**1→6]**C**1→4**B**1→6**R** sequence for compound **6a**, that is, α-D-Glc*p*-(1→4)-[α-D-Glc*p*-(1→6)-]α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→6)-D-Glc*p* (Scheme 1). It is interesting to note that branching has a specific influence on the position of the H-1 signal of the branched

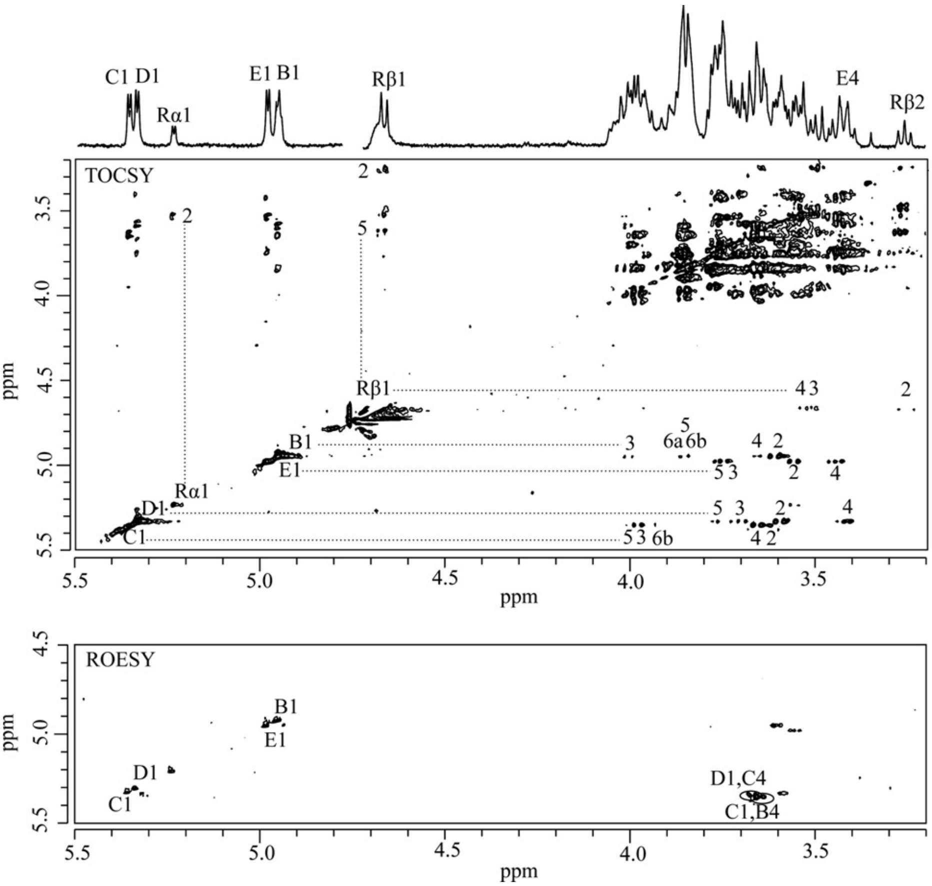


Figure 5. 500-MHz 1D ^{1}H NMR spectrum, 2D ^{1}H–^{1}H TOCSY spectrum (mixing time 180 ms) and 2D ^{1}H–^{1}H ROESY spectrum (mixing time 300 ms) of fraction **6a**, recorded at 300 K in D_2O. For an explanation of the coding systems, see Figure 4.

residue: δ 5.399 for -(1→4)-α-D-Glc*p*-(1→4)- (maltotriose)[8] and δ 5.361 for -(1→4,6)-α-D-Glc*p*-(1→4)- (compound **6a**). Remarkably, the downfield shift of H-5 when going from -(1→4)-α-D-Glc*p*- to -(1→4,6)-α-D-Glc*p*- in the case of residue **C** ($\Delta\delta$ +0.20 ppm) is similar to that observed for residue **Rα** in **4a** ($\Delta\delta$ +0.17 ppm).

The established structure of the pentasaccharide was verified by 2D ^{1}H–^{1}H ROESY measurements (Fig. 5), showing inter-residual cross-peaks between **C** H-1 and **B** H-4, and between **D** H-1 and **C** H-4. Inter-residual cross-peaks for **B** H-1 and **E** H-1 were not observed, due to low signal intensity.

2.2.7. Fraction 7.

The MALDI-TOF mass spectrum of fraction **7** revealed $[M+Na]^+$ pseudomolecular ions at m/z 851 and 1013, corresponding with Hex_5 and Hex_6, respectively. Fraction **7** was further separated on CarboPac PA-1 (eluent: 100 mM NaOAc in 100 mM NaOH) (Fig. 2C), yielding major fraction **7a** (Hex_6) and minor fraction **7b** (Hex_5).

The 1D ^{1}H NMR spectrum of **7a** (Fig. 3C) showed anomeric signals at δ 5.387 (**A**$^{\mathrm{I}}$ H-1, $^3J_{1,2}$ 3.9 Hz), 5.380 (**A**$^{\mathrm{II}}$ H-1, $^3J_{1,2}$ 3.9 Hz), 5.239 (**Rα** H-1, $^3J_{1,2}$ 3.6 Hz), 4.955 (**B**$^{\mathrm{I,II}}$ H-1 and **E** H-1, $^3J_{1,2}$ 3.7 Hz) and 4.669 (**Rβ** H-1, $^3J_{1,2}$ 7.8 Hz). Table 1 includes the chemical shifts of the various residual protons as determined by 2D ^{1}H–^{1}H TOCSY analysis (not shown). In TOCSY experiments with different mixing times (data not shown) the built-up of the scalar coupling network could be followed. The set of H-1α and H-1β chemical shifts of the reducing residue **R** corresponds with the value of a -(1→6)-D-Glc*p* unit.[8] The **E** H-4 signal at δ 3.432 (dd, 1H) corresponds with a terminal α-D-Glc*p*-(1→6)- unit (compare with compounds **3a**, **4a**, **5a** and **6a**), thereby indicating a linear structure. Taking into account the methylation analysis data of **EPS35-5**, the **A** H-1 signals at δ 5.387 and 5.380 reflect the occurrence of two (-)α-D-Glc*p*-(1→4)- units, denoted by **A**$^{\mathrm{I}}$ and **A**$^{\mathrm{II}}$.[8] The chemical shift patterns of **A**$^{\mathrm{I}}$ and **A**$^{\mathrm{II}}$ are the same and fit best with the values found for residue **A** in compound **5a**, indicating two -(1→6)-α-D-Glc*p*-(1→4)- units in compound **7a** (**A** H-5 at δ 3.92). The surface area of the **B** and **E** H-1 signals at δ 4.955 matches 3H, indicating the presence of three (-)α-D-Glc*p*-(1→6)- units. In the TOCSY experiments with incremental mixing times (data not shown), two sets of chemical shifts could be observed. The set **B** H-2, H-3 and H-4 at δ 3.60, 4.01 and 3.66, respectively, matches the values of a -(1→4)-α-D-Glc*p*-(1→6)- unit (see compounds **3a**, **5a** and **6a**). Residue **E** showed the typical proton chemical shift pattern of a terminal α-D-Glc*p*-(1→6)- unit (see above). Combination of the various data indicates that the hexasaccharide contains twice the **X**1→4**B**1→6**Y** sequence and twice the **X**′1→6**A**1→4**Y**′ sequence. With residue **R** in an **X**″1→6**R** sequence and **E** as a terminal unit, these data lead to a **E**1→6**A**^{II}1→4**B**^{II}1→6**A**^{I}1→4**B**^{I}1→6**R** sequence for **7a**, that is, α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→6)-D-Glc*p* (Scheme 1).

The structure of fraction **7a** was verified by 2D ^{1}H–^{1}H ROESY measurements (not shown), showing inter-residual cross-peaks between **A** H-1 and **B** H-4, between **B** H-1 and **A** H-6a and **R** H-6a, and between **E** H-1 and **A** H-6a.

1D ^{1}H NMR analysis of fraction **7b** (Fig. 6) revealed anomeric signals at δ 5.381 (**A**$^{\mathbf{I,II}}$ H-1, $^3J_{1,2}$ 3.8 Hz), 5.225 (**Rα** H-1, $^3J_{1,2}$ 3.6 Hz), 4.954 (**B** H-1 and **E** H-1, $^3J_{1,2}$ 3.8 Hz) and 4.649 (**Rβ** H-1, $^3J_{1,2}$ 8.0 Hz). The chemical shift values as determined by 2D ^{1}H–^{1}H TOCSY spectroscopy (Fig. 6/180 ms) are shown in Table 1. The set of anomeric signals of **Rα** and **Rβ** at δ 5.225 and 4.649, respectively, corresponds with the occurrence of a -(1→4)-D-Glc*p* unit.[8] Similar to compound **7a**, the two **A** residues of **7b** that overlap on the **A**$^{\mathbf{I,II}}$ TOCSY track develop exactly the same built-up chemical shift patterns in the TOCSY experiments with different mixing times (data not shown), demonstrating the presence of two -(1→6)-α-D-Glc*p*-(1→4)- units. Note also the similarity with residue **A** in compound **5a**. The H-1 signal at δ 4.954 (2H) indicates the occurrence of two (-)α-D-Glc*p*-(1→6)- units, that is, residues **B** and **E**. In TOCSY experiments with incremental mixing times (data not shown) two distinctly different proton chemical shift patterns develop. The **B** H-3 resonance is observed at δ 4.00, indicating a -(1→4)-α-D-Glc*p*-(1→6)- unit. On guidance of the structural-reporter-group concept, the **E** H-4 signal at δ 3.429 identified residue **E** as a terminal α-D-Glc*p*-(1→6)- unit. With twice the **X**1→6**A**1→4**Y** sequence and a 4-substituted **R** unit, it can be concluded that compound **7b** has a **E**1→6**A**^{II}1→4**B**1→6**A**^{I}1→4**R** sequence, that is, α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc*p* (Scheme 1).

The sequence established for pentasaccharide **7b** was verified by 2D ^{1}H–^{1}H ROESY measurements (Fig. 6), revealing inter-residual cross-peaks between **A** H-1 and

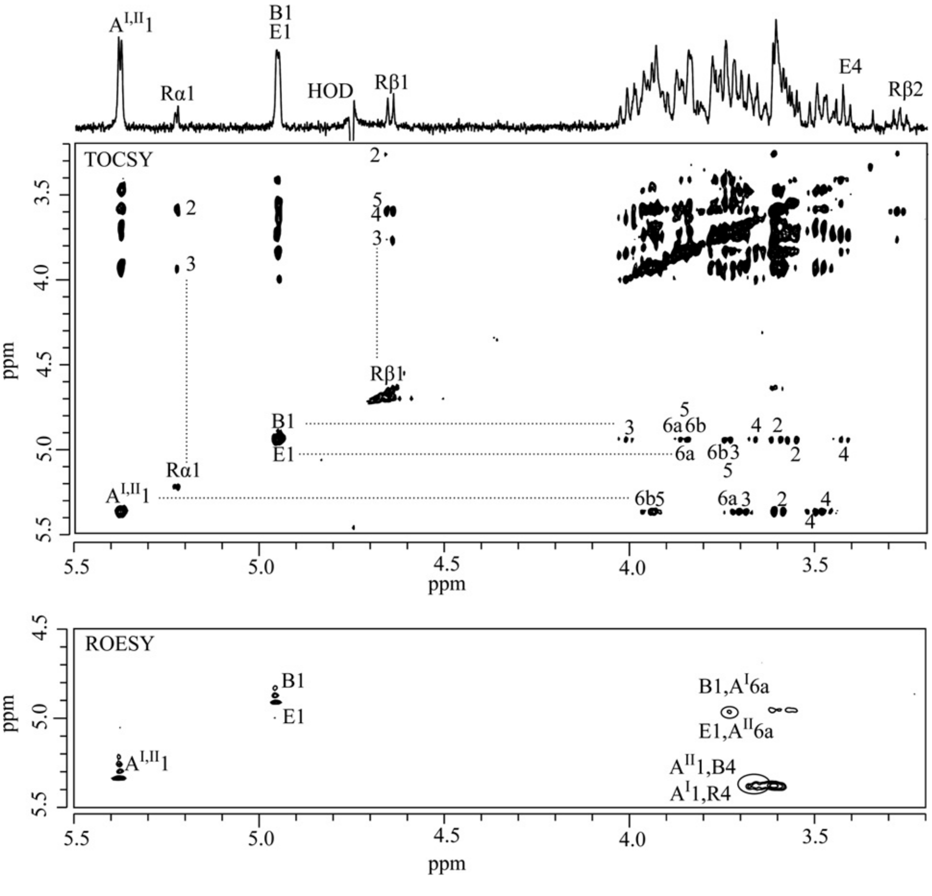


Figure 6. 500-MHz 1D ^{1}H NMR spectrum, 2D ^{1}H–^{1}H TOCSY spectrum (mixing time 180 ms) and 2D ^{1}H–^{1}H ROESY spectrum (mixing time 300 ms) of fraction **7b**, recorded at 300 K in D$_2$O. For an explanation of the coding systems, see Figure 4.

B H-4 and **Rβ** H-4, between **B** H-1 and **A**^{I} H-6a, and between **E** H-1 and **A**^{II} H-6a.

2.3. 2D NMR spectroscopy of EPS35-5

In order to unravel the 1D ^{1}H NMR spectrum of **EPS35-5** (Fig. 1), 2D TOCSY experiments with increasing mixing times of 10, 30, 60, 120 and 180 ms, as well as data from the ^{13}C–^{1}H HSQC measurements of **EPS35-5** were interpreted (Table 1) using the data collected from the oligosaccharides obtained by partial acid hydrolysis of **EPS35-5** (Section 2.2).

In the 60 ms TOCSY spectrum (Fig. 7), the **A/D** H-1 track at δ 5.36 (indicating (α1→4)-linked D-Glc*p*)[8] showed one chemical shift value for H-2 at δ 3.60 and one value for H-3 at δ 3.70, indicating that these residues are not 4-substituted (library data from maltotriose: $\delta_{\text{H-2}}$ 3.63 and $\delta_{\text{H-3}}$ 3.96).[8] In the 120 ms TOCSY spectrum (Fig. 7) the **A/D** H-1 track revealed two different H-4 signals at δ 3.48 (residue **A**), indicating -(1→6)-α-D-Glc*p*-(1→4)- units (see residue **A** in **5a**, **7a** and **7b**), and 3.42 (residue **D**), indicating terminal α-D-Glc*p*-(1→4)- units (see residue **D** in **3a**, **4a** and **6a**).

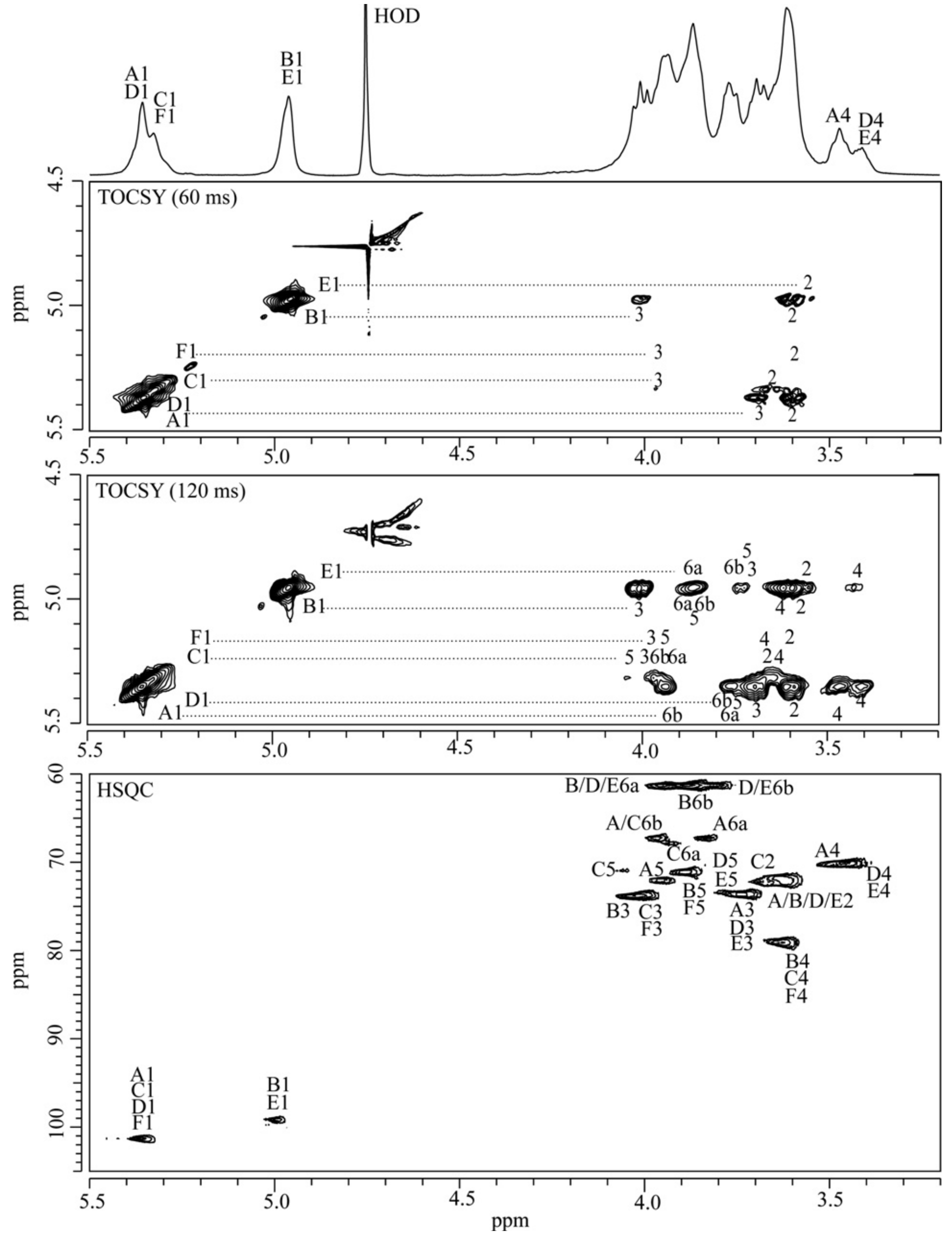


Figure 7. 500-MHz 1D ^{1}H NMR spectrum, 2D ^{1}H–^{1}H TOCSY spectra (top, mixing time 60 ms; bottom, 120 ms) and 2D ^{13}C–^{1}H HSQC spectrum of **EPS35-5** recorded at 300 K in D_2O. Anomeric protons in the TOCSY spectrum (A1, etc.) have been indicated on the diagonal; numbers in the horizontal tracks belong to the cross-peaks of the scalar-coupling network of the residues indicated. In the ^{13}C–^{1}H HSQC spectrum A1 denotes the cross-peak between H-1 and C-1 of residue **A**, etc.

The **C**/**F** H-1 track in the 60 ms TOCSY spectrum at δ 5.33 (indicating (α1→4)-linked D-Glc*p* residues) showed two different H-2 signals for residues **C** and **F** at δ 3.65 (compare with residue **C** in **6a**) and 3.60, respectively, reflecting different substitution patterns (Scheme 1). The H-3 signal, however, is observed at δ 3.98 for both residues, indicating the occurrence of -(1→4)-α-D-Glc*p*-(1→4)- units. The **C** H-5 resonance at δ 4.05 fits with the occurrence of a -(1→4,6)-α-D-Glc*p*-(1→4)- unit (see residue **C** in **6a**).

On the **B**/**E** H-1 track in the 60 ms TOCSY spectrum at δ 4.96 (indicating (α1→6)-linked D-Glc*p* residues) two different H-2 signals are observed, a strong signal at δ 3.61 (residue **B**) and a weak signal at δ 3.56 (residue **E**). The set of **B** H-2 and H-3 at δ 3.61 and 4.01 corresponds with the occurrence of a -(1→4)-α-D-Glc*p*-(1→6)- unit (compare with residue **B** in **3a**, **5a**, **6a**, **7a** and **7b**). In the 120 ms TOCSY spectrum residue **E** H-4 is observed at δ 3.43, in agreement with a terminal α-D-Glc*p*-(1→6)- unit. Compare also the other chemical shift values with those found for residue **E** in compounds **4a**, **5a**, **6a**, **7a** and **7b** (Table 1). On the same track the set of **B** H-4, H-5, H-6a and H-6b supports the occurrence of a -(1→4)-α-D-Glc*p*-(1→6)- unit. The absence of a H-4 cross-peak at δ 3.50 indicates that a -(1→6)-α-D-Glc*p*-(1→6)- element does not occur in **EPS35-5**.[8,9] The fact that the H-6a and H-6b values on the (α1→6) anomeric track exclude 6-substitution, further confirms the conclusion that only single (α1→6) bridges occur in **EPS35-5**.

The substitution patterns established from the TOCSY spectra were verified using ^{13}C–^{1}H HSQC spectroscopy (Fig. 7), yielding $\delta_{\text{C-4}}$ values of 78.9 ppm for residues **B**, **C** and **F**, reflecting the 4-substitution of these residues.[11] Residues **A** and **C** gave rise to $\delta_{\text{C-6}}$ values of 67.1 ppm, reflecting the 6-substitution of these residues.[11] For the residues **B**, **D** and **E** the $\delta_{\text{C-6}}$ value of 61.3 ppm (**F** was not detected, due to minor occurrence) confirmed the absence of a 6-substitution.

2.4. Enzymatic hydrolysis

The occurrence of both (α1→4) and (α1→6) linkages in **EPS35-5** may render the polysaccharide susceptible to hydrolysis by *Klebsiella planticola* pullulanase M1, which specifically cleaves the (α1→6) linkage in (-)α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→6)- sequences.[12] The substrate specificity of the commercially available enzyme was checked using a series of model compounds (data not shown).

A sample of **EPS35-5** (20 mg) was incubated with pullulanase M1, and the initial progress of the hydrolysis was followed by analysing aliquots at 30, 60, 90 and 120 min by MALDI-TOF-MS and 1D ^{1}H NMR spectroscopy. As clear indications were obtained for the formation of oligosaccharide fragments (MALDI-TOF-MS), accompanied by a decrease in the anomeric signal of (-)α-D-Glc*p*-(1→6)- units ($\delta_{\text{H-1}}$ 4.96; 1D ^{1}H NMR spectroscopy), the hydrolysis was continued for 96 h. The resulting mixture was analysed by MALDI-TOF-MS, revealing $[\text{M+Na}]^+$ pseudomolecular ions at *m*/*z* 527, 689 and 851, corresponding with Hex$_3$, Hex$_4$ and Hex$_5$, respectively. An aliquot of the mixture was analysed on CarboPac PA-100 (Fig. 8), using 0–300 mM NaOAc in 100 mM NaOH as eluent; for identification and quantification purposes a mixture of maltose, maltotriose, panose, maltotetraose, maltopentaose and maltohexaose standards was subjected to the same pullulanase hydrolysis and desalting procedure as the **EPS35-5** sample, and also analysed on CarboPac PA-100, using 0–300 mM NaOAc in 100 mM NaOH as eluent. The bulk of the pullulanase M1 hydrolysate was separated on CarboPac PA-1, using the same elution conditions, yielding fractions **P1** to **P6**.

2.4.1. Fraction P1.

HPAEC and 1D ^{1}H NMR analysis showed fraction **P1** to contain maltose,[8] α-D-Glc*p*-(1→4)-D-Glc*p*. Using the response factor of maltose in the standard mixture, the relative amount of **P1** in the hydrolysate was determined to be 51 mol % (Scheme 1).

2.4.2. Fraction P2.

MALDI-TOF-MS analysis of fraction **P2** revealed an $[\text{M+Na}]^+$ pseudomolecular ion at *m*/*z* 527, corresponding with Hex$_3$. The retention time of **P2** on CarboPac PA-100 matched that of panose, whereas the 1D ^{1}H NMR spectrum of **P2** corresponded with panose,[8] α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc*p*. Using the response factor of panose in the standard mixture, the relative amount of **P2** was determined to be 7 mol % (Scheme 1).

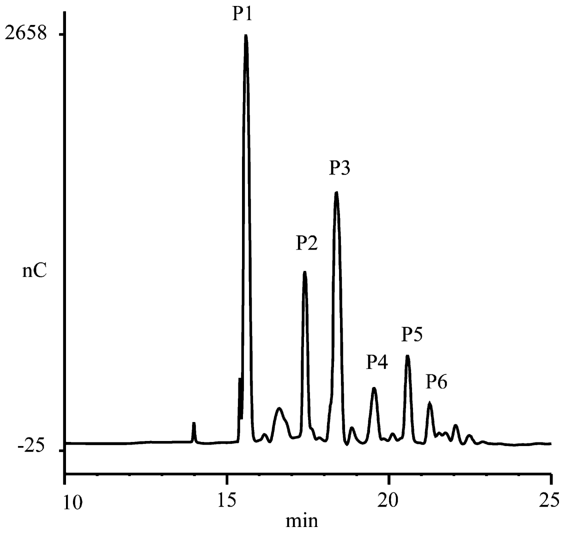


Figure 8. HPAEC-PAD profile of pullulanase M1 hydrolysate on CarboPac PA-100, using a linear gradient.

2.4.3. Fraction P3.

The MALDI-TOF mass spectrum of fraction **P3** showed an $[M+Na]^+$ pseudomolecular ion at *m/z* 527, corresponding with Hex_3. The retention time of **P3** on CarboPac PA-100 matched that of maltotriose, and the 1D ^{1}H NMR spectrum of **P3** also corresponded with that of maltotriose, α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-D-Glc*p*.[8] The amount of **P3** was quantified, using the response factor of maltotriose, yielding 31 mol % (Scheme 1).

2.4.4. Fraction P4.

MALDI-TOF-MS analysis of fraction **P4** revealed a single $[M+Na]^+$ pseudomolecular ion at *m/z* 689, corresponding with Hex_4. Using the response factor of maltotetraose, the relative amount of **P4** was estimated to be 3 mol %. The 1D ^{1}H NMR spectrum of **P4** indicated the presence of a mixture of two components in a 2:1 ratio. Fraction **P4** was further separated on CarboPac PA-1, using 100 mM NaOAc in 100 mM NaOH, yielding a major fraction **P4a** (Hex_4) and a minor fraction **P4b** (Hex_4).

The 1D ^{1}H NMR spectrum of **P4a** (Fig. 3D) showed 5 anomeric signals at δ 5.363 (**C** H-1, $^3J_{1,2}$ 3.2 Hz), 5.340 (**D** H-1, $^3J_{1,2}$ 3.2 Hz), 5.226 (**Rα** H-1, $^3J_{1,2}$ 3.2 Hz), 4.985 (**E** H-1, $^3J_{1,2}$ 3.2 Hz) and 4.655 (**Rβ** H-1, $^3J_{1,2}$ 7.9 Hz). The set of anomeric signals of **Rα** and **Rβ** corresponds with the occurrence of a reducing -(1→4)-D-Glc*p* unit.[8] It is clear that one (α1→6)-linked and two (α1→4)-linked D-Glc*p* residues are present in Hex_4. The H-1 signals of residues **C**, **D** and **E** fit with the values observed in compound **6a** for the **D**1→4[**E**1→6]**C** branch-point. Note the two overlapping H-4 signals at δ 3.41 and 3.43, corresponding with the occurrence of both α-D-Glc*p*-(1→4)- and α-D-Glc*p*-(1→6)- units. These data lead to the sequence **D**1→4[**E**1→6]**C**1→4**R** for **P4a**, that is, α-D-Glc*p*-(1→4)-[α-D-Glc*p*-(1→6)-]α-D-Glc*p*-(1→4)-D-Glc*p* (Scheme 1).

The 1D ^{1}H NMR spectrum of **P4b** (Fig. 3E) revealed two (-)α-D-Glc*p*-(1→4)- units (residues **A** and **F**, δ_{H-1} 5.382, 2H) and one (-)α-D-Glc*p*-(1→6)- unit (residue **E**, δ_{H-1} 4.944, 1H). Furthermore, only one H-4 signal of a terminal residue at δ 3.43 (residue **E**) is observed, indicating a linear structure. The reducing residue **R** H-1α and H-1β resonances at δ 5.225 and 4.650, respectively, correspond with the occurrence of a reducing -(1→4)-D-Glc*p* unit. These data lead to an **E**1→6**A**1→4**F**1→4**R** sequence for the linear tetrasaccharide **P4b**, that is, α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-D-Glc*p* (Scheme 1).

2.4.5. Fraction P5.

The MALDI-TOF-MS spectrum of fraction **P5** showed an $[M+Na]^+$ pseudomolecular ion at *m/z* 689, corresponding with Hex_4. The retention time of **P5** on CarboPac PA-100, as well as its 1D ^{1}H NMR spectrum, matched that of maltotetraose, α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-D-Glc*p* (Scheme 1). Using the response factor of the maltotetraose standard, the relative amount of **P5** was determined to be 6 mol %.

2.4.6. Fraction P6.

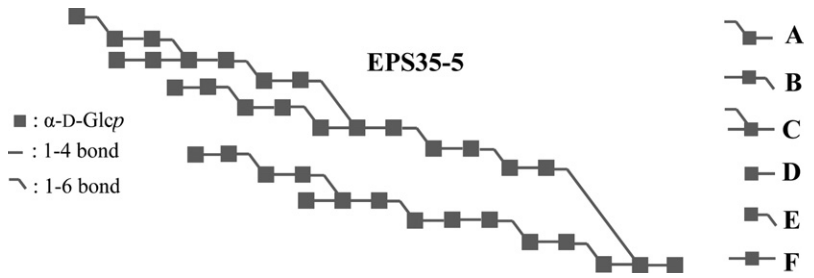
Fraction **P6** was analysed by MALDI-TOF-MS, revealing an $[M+Na]^+$ pseudomolecular ion at *m/z* 851, corresponding with Hex_5. The retention time of **P6** on CarboPac PA-100 did not match that of maltopentaose, which eluted later than **P6**, indicating a structure with at least one (α1→6) linkage. Using the response factor of maltopentaose the relative abundance of **P6** was estimated to be 2 mol %. Fraction **P6** did not contain sufficient material for further analysis.

2.4.7. Evaluation of the pullulanase M1 incubation.

Based on MALDI-TOF-MS (not shown) and HPAEC-PAD (Fig. 8) analysis data, it can be concluded that the pullulanase M1 hydrolysis of **EPS35-5** is complete. When taking into account the implicit (α1→6) linkage at the reducing side of the compounds presented in Scheme 1, the linkage distribution represented by these oligosaccharides in their abundances matches the linkage distribution determined by methylation analysis as well as 1D ^{1}H NMR data of full length **EPS35-5**, thereby confirming the completeness of the enzymatic hydrolysis. The various identified fragments describe all structural elements present in **EPS35-5**.

3. Conclusions

A composite model, that includes all identified structural features of the native **EPS35-5** α-D-glucan was formulated by combining ^{1}H NMR data of **EPS35-5** with methylation analysis data (Section 2.1), as well as with structural information of oligosaccharides obtained by partial acid hydrolysis (Section 2.2) and pullulanase M1 hydrolysis (Section 2.4) of **EPS35-5**, and is depicted in Scheme 2. The methylation analysis data, combined with 1D ^{1}H NMR surface areas and pullulanase M1 fragment quantities dictate the boundaries for the distribution of the six types of building blocks in **EPS35-5** (Table 2). All oligosaccharides obtained by partial acid hydrolysis and pullulanase M1 hydrolysis of **EPS35-5** (Scheme 1) fit into the picture, reflecting the validity of the representation. It can be concluded that the (1→4, 1→6)-α-D-glucan of *Lactobacillus reuteri* strain 35-5 has a heterogeneous structure with no repeating units present. It is a built-up from maltose, maltotriose and maltotetraose elements with single (α1→6) bridges. The majority of the terminal residues are α-D-Glc*p*-(1→4)- units, however, a small amount of α-D-Glc*p*-(1→6)- units also occurs. The significant occurrence of compound **P1** leads to a structure with large amounts of alternating (α1→4) and (α1→6) linkages. It should be noted that the advantage of constructing composite models is that α-D-glucans isolated from different



Scheme 2. Composite model of the native **EPS35-5** α-D-glucan. The composite takes into account all facts from the methylation analysis of **EPS35-5** and the various ^1H NMR analyses of **EPS35-5** and its established fragments. Residue labels correspond with those used in the text, tables and figures, and are presented on the right.

Table 2. Percentages of building blocks **A**–**F** present in **EPS35-5**

Building block	Code	Abundance (%)
-(1→6)-α-D-Glc*p*-(1→4)-	**A**	28
-(1→4)-α-D-Glc*p*-(1→6)-	**B**	38
-(1→4,6)-α-D-Glc*p*-(1→4)-	**C**	14
α-D-Glc*p*-(1→4)-	**D**	10
α-D-Glc*p*-(1→6)-	**E**	4
-(1→4)-α-D-Glc*p*-(1→4)-	**F**	6

bacterial sources or prepared from sucrose by mutant glucansucrases can easily be compared.

4. Materials and methods

4.1. Materials

EPS35-5 was a gift from TNO Quality of Life, Zeist, The Netherlands. D_2O (99.9 atom %) was purchased from Cambridge isotope laboratories, Inc., Andover, MA. A suspension of pullulanase M1 (*Klebsiella planticola*) in 3.2 M $(NH_4)_2SO_4$ was purchased from Megazyme International Ireland Ltd, Bray, Ireland.

4.2. Monosaccharide analysis

A polysaccharide sample was subjected to methanolysis (1.0 M methanolic HCl, 24 h, 85 °C), followed by re-N-acetylation and trimethylsilylation (1:1:5 hexamethyldisilazane–trimethylchlorosilane–pyridine; 30 min, room temperature). The mixture of trimethylsilylated methyl glycosides was analysed by GLC on an EC-1 column (30 m × 0.32 mm, Alltech Associates Inc., Illinois, USA) using a Chrompack CP 9002 gas chromatograph (temperature gradient, 140–240 °C at 4 °C/min). The identification of the monosaccharide derivatives was confirmed by GLC–EI-MS.[13]

4.3. Linkage analysis

A polysaccharide sample was permethylated using CH_3I and solid NaOH in DMSO, as described earlier.[14] After hydrolysis with 2 M TFA (2 h, 120 °C), the partially methylated monosaccharides were reduced with $NaBD_4$ (2 h, room temperature). Conventional work-up, involving neutralisation with HOAc and removal of boric acid by co-evaporation with MeOH, followed by acetylation with 1:1 Ac_2O–pyridine (3 h, 120 °C), yielded a mixture of partially methylated alditol acetates, which was analysed by GLC–EI-MS.[13,15]

4.4. Partial acid hydrolysis

In pilot experiments, 5-mg aliquots of **EPS35-5** were hydrolysed for 30, 60, 90 and 120 min (0.5 M TFA, 90 °C). After concentration under reduced pressure, the residues were analysed by MALDI-TOF-MS and 1D ^1H NMR spectroscopy. Based on the initial results, **EPS35-5** (500 mg) was hydrolysed in 25 mL of 0.5 M TFA for 2 h at 90 °C. The solution was concentrated under reduced pressure at a rotary evaporator, and the residue was separated on a Bio-Gel P-4 column (400 × 12 mm, BioRad), eluted with 25 mM NH_4HCO_3; 0.9-mL fractions were collected at a flow rate of 13.5 mL/h. Fractions were tested for the presence of carbohydrate by a TLC spot-test with orcinol/H_2SO_4 staining. Carbohydrate-containing fractions were analysed by MALDI-TOF-MS.

4.5. Enzymatic hydrolysis

An equimolar mixture of maltose, maltotriose, panose, maltotetraose, maltopentaose and maltohexaose (50 mM, 1 mL) was dissolved in assay buffer (50 mM NaOAc, pH 5.0 with 4 M HOAc), and subsequently 5 μL pullulanase M1 suspension was added. A sample of 20 mg **EPS35-5** was dissolved in 2 mL assay buffer (see above), and 30 μL pullulanase M1 suspension was added. Hydrolysis of samples was carried out for 96 h at 37 °C. From each sample aliquots were taken at $t = 30$, 60, 90 and 120 min, and subsequently boiled for 2 min to terminate enzymatic hydrolysis. Each sample was desalted on CarboGraph SPE columns (150 mg graphitised carbon, Alltech) using 1:3 acetonitrile–H_2O as eluent, lyophilised, and subsequently analysed by

HPAEC-PAD, MALDI-TOF-MS and 1D ^{1}H NMR spectroscopy. The bulk of the **EPS35-5** sample was finally desalted, lyophilised and re-dissolved in 300 μL H_2O. Pullulanase hydrolysis fragments were separated by HPAEC and analysed by MALDI-TOF-MS and 1D ^{1}H NMR spectroscopy.

4.6. HPAEC-PAD

High-pH anion-exchange chromatography was performed on a Dionex DX500 workstation, equipped with an ED40 pulsed amperometric detection (PAD) system. A triple-pulse amperometric waveform (E_1 0.1 V, E_2 0.7 V, E_3 −0.1 V) was used for detection with the gold electrode.[16] Analytical separations were performed on a CarboPac PA-100 column (250 × 4 mm, Dionex), using a linear gradient of 0–300 mM NaOAc in 100 mM NaOH (1 mL/min). Samples were fractionated on a CarboPac PA-1 column (250 × 9 mm, Dionex), using a linear gradient of 0–300 mM NaOAc in 100 mM NaOH (4 mL/min) or isocratic conditions of 100 mM NaOAc in 100 mM NaOH (4 mL/min). Collected fractions were immediately neutralised with 4 M HOAc, desalted on CarboGraph SPE columns (150 mg graphitised carbon, Alltech) using 1:3 acetonitrile–H_2O as eluent and lyophilised.

4.7. Mass spectrometry

GLC–EI-MS was performed on a Fisons Instruments GC 8060/MD 800 system (Interscience BV; Breda, The Netherlands) equipped with an AT-1 column (30 m × 0.25 mm, Alltech), using a temperature gradient of 140–240 °C at 4 °C/min.[13]

Matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) was carried out on a Voyager-DE Pro (Applied Biosystems; Nieuwerkerk aan de IJssel, The Netherlands) instrument in the reflector mode at an accelerating voltage of 24 kV, using an extraction delay of 90 ns, in a resolution of 5000–9000 FWMH. Samples (1 μL) were mixed in a 1:1 ratio with a mixture of 7.5 mg/mL 2,5-dihydroxybenzoic acid (DHB) in 1:1 acetonitrile–H_2O, and spectra were recorded in the positive-ion mode.

4.8. NMR spectroscopy

^{1}H NMR spectra, including ^{1}H–^{1}H and ^{13}C–^{1}H correlation spectra, were recorded on a Bruker DRX500 spectrometer (Bijvoet Center, Department of NMR spectroscopy, Utrecht University) at a probe temperature of 300 K. Samples were exchanged once with 99.9 atom% D_2O, lyophilised and dissolved in 650 μL D_2O. ^{1}H chemical shifts (δ) are expressed in ppm by reference to internal acetone (δ 2.225), and ^{13}C chemical shifts (δ) are expressed in ppm by reference to the methyl-carbon of internal acetone (δ 31.08). 1D ^{1}H NMR spectra were recorded with a spectral width of 5000 Hz in 16k complex data sets and zero filled to 32k. A WEFT pulse sequence was applied to suppress the HOD signal.[17] When necessary, a fifth order polynomial baseline correction was applied. 2D TOCSY spectra were recorded using MLEV17 mixing sequences with spin-lock times of 10, 30, 60, 120 and 180 ms. The spin-lock field strength corresponded to a 90° pulse width of about 28 μs at 13 dB. The spectral width in 2D TOCSY experiments was 4006 Hz at 500 MHz in each dimension. 400–1024 spectra of 2k data points with 8–32 scans per t_1 increment were recorded. 2D rotating-frame nuclear Overhauser enhancement spectra (ROESY) were recorded with 300 ms mixing time. The spectral width was 4006 Hz at 500 MHz in each dimension. Suppression of the HOD signal was performed by 1 s pre-saturation during the relaxation delay. Between 400 and 1024 data sets of 2k data points were recorded with 8–16 scans per t_1 increment. 2D ^{13}C–^{1}H HSQC spectroscopy was carried out at a ^{1}H frequency of 500.0821 MHz and a ^{13}C frequency of 125.7552 MHz. Spectra were recorded with a spectral width of 4006 Hz for t_2 and 10 kHz for t_1. The HOD signal was pre-saturated for 1 s, and ^{12}C-bound protons were suppressed using a TANGO pulse sequence. During acquisition of the ^{1}H FID, a ^{13}C decoupling pulse was applied. 128–256 experiments of 2k data points were recorded with 128 scans per t_1 increment. 2D NMR spectroscopic data were analysed by applying a sinus multiplication window and zero filling to spectra of 4k by 1k dimensions. In the case of ^{13}C–^{1}H HSQC data, the spectra were zero filled to 4k by 512 data points. A Fourier transform was applied, and where necessary, a fifth to fifteenth order polynomial baseline function was applied. All NMR data were processed using in-house developed software (J.A. van Kuik, Bijvoet Center, Department of Bio-Organic Chemistry, Utrecht University).

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